Role of Glutathione in the Hypoxic Cell Cytotoxicity of Misonidazole

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ABSTRACT

Misonidazole (MIS) is a hypoxic cell radiosensitizer currently undergoing Phase III clinical trials in the treatment of cancer by radiation. It is also a cytotoxic agent with specificity toward hypoxic cells, and consequently has a tumoricidal effect in laboratory animals. This tumoricidal effect has not been clinically applicable, in part because the initial resistance to the cytotoxic action of MIS (the nonexponential portion, or shoulder, of the semilogarithmic plot of the surviving fraction of the cell population versus the time of exposure to MIS, referred to hereafter as the shoulder of the survival curve) for cells treated with MIS under hypoxic conditions is too large to be overcome at clinically tolerable doses of MIS. We report here that pretreatment of Chinese hamster ovary cells in vitro with diethylmaleate to deplete intracellular glutathione results in a substantial decrease in the shoulder of the survival curve for MIS-treated hypoxic cells. Restoration of glutathione results in restoration of the shoulder of the survival curve and a slight extension beyond that seen with control cells. These results demonstrate that glutathione protects against the cytotoxic effect of MIS. However, glutathione depletion does not significantly affect the rate of binding of MIS metabolites to cellular macromolecules, indicating that the cytotoxicity of MIS is not simply a reflection of massive binding of MIS metabolites to cellular constituents. We propose that the cytotoxicity of MIS toward hypoxic cells is a result of hydrogen abstraction from target molecules by free radicals formed in the reduction of the nitro group.

INTRODUCTION

MIS is currently undergoing clinical trials as a hypoxic cell radiosensitizer (16, 45). The mechanism of radiosensitization of hypoxic cells by electron-affinic radiosensitizers, such as MIS, is thought to involve competition with endogenous thiols for radiation-induced radicals (9, 46), a process which depends only on the concentration of radiosensitizer at the time of irradiation. However, prolonged exposure to MIS under hypoxia leads to biochemical alterations which render the cells more sensitive to X-rays, even after washing them free of MIS (47). This hypoxic "preincubation effect" also sensitizes cells to the cytotoxic action of a number of chemotherapeutic agents in vitro (35). Sensitization of tumor cells in vivo to chemotherapy has also been observed (13, 33, 36), and a therapeutic gain for combination chemotherapy with MIS has been reported, even at clinically achievable doses (5).

MIS is also preferentially toxic to hypoxic cells (23, 32). This effect is of potential clinical interest, since MIS can kill a large percentage of the cells in various animal tumors in vivo, apparently because of the hypoxic cells in these tumors, since small lung metastases, which have not developed hypoxic regions, are not affected (4). The clinical usefulness of these observations is limited by the neurotoxicity of MIS, which occurs in humans at lower doses than are required for a substantial tumoricidal effect in mice (14–16). Nevertheless, neurotoxicity may differ mechanistically from toxicity to tumor cells, and it may therefore be possible to enhance the tumoricidal effect of MIS without increasing its neurotoxicity.

The preferential cytotoxicity of MIS to hypoxic cells has been attributed to the metabolic reduction of the nitro group, which is suppressed by oxygen:

\[
R—\text{NO}_2 \rightarrow R—\text{NO}^- \rightarrow R—\text{N}=\text{O} \rightarrow R—\text{N}=\text{OH} \rightarrow R—\text{N}^- \rightarrow R—\text{NH}_2
\]

This metabolic scheme (2) is common to a number of nitroheterocyclic compounds. The cytotoxicity of these nitroheterocyclic compounds generally increases with increasing one-electron reduction potential (1), which is a measure of the ability of the nitro group to accept an electron to form the nitroanion radical. The fact that the conditions necessary for cytotoxicity are the same as those required for reductive metabolism (such as hypoxia and \( \text{pH} \)) has led to the belief that one or more of the metabolites formed are responsible for cytotoxicity. It has been postulated that the mechanism of cytotoxic action involves irreversible binding of these metabolites to the target molecules (31, 41) since binding also occurs under conditions conducive to reduction of the nitro group. However, none of the reduced metabolites has been definitively identified as the toxic intermediate.

The toxicity of MIS toward hypoxic cells can be modified by a variety of agents. Ascorbate enhances the cytotoxicity of MIS, apparently by accelerating its reductive metabolism (27, 39). Thiols protect against MIS cytotoxicity (22, 27, 39). Serum and pH also play a role in modifying MIS cytotoxicity, although these effects may be related to thiol perturbation (26). The effect of thiols is largely on the shoulder of the MIS cytotoxicity curve. Since MIS has been shown to deplete intracellular non-protein thiols under hypoxic conditions (44), it has been suggested (39) that the shoulder of the MIS cytotoxicity curve for hypoxic cells is a result of the protective effect of endogenous thiols, which is lost as these thiols are depleted.

We have investigated the importance of GS, which is the main non-protein thiol in mammalian cells (24), in the expression of MIS cytotoxicity, by pretreating CHO cells with DEM, a reagent which has been used to lower GS levels in vitro (6, 8, 37) and in vivo (3). The reaction between GSH and DEM is catalyzed by GSH S-transferase (11, 12), which is widely...
distributed in mammalian tissues (10). The reaction also occurs nonenzymatically (3). The use of DEM as a reagent to deplete intracellular GS is discussed in detail in a recent review (7).

Since covalent binding of reactive intermediates of MIS metabolism to cellular macromolecules has been suggested as a possible mechanism of cytotoxic action of MIS, we have measured the rate of this binding in normal and glutathione-depleted cells to determine its importance in the killing of hypoxic cells by MIS.

MATERIALS AND METHODS

Drug Treatment. DEM (technical grade, 90 to 96%) was obtained from Sigma Chemical Co., St. Louis, Mo., and was found to be stable for several months at 0–5°C, with gradual isomerization to diethyl fumarate (20% isomerization after 6 months). Solutions for in vitro experiments were prepared by sonication in 0.9% NaCl solution. All other reagents were of the highest grade commercially available. MIS was obtained from the Drug Synthesis and Chemistry Branch of the National Cancer Institute. [2-'4C]MIS (15.6 mCi/mmol) was obtained from the Pharmaceutical Resources Branch of the National Cancer Institute.

Assays for Intracellular Thiols. GSH and GSSG were assayed by a minor modification of the enzymatic method of Tietze (40), which consists of cyclic reduction of GSSG with GS reductase and NADPH, and concurrent reduction of the colorimetric reagent DTNB. GSH and GSSG can be differentiated by alkylation of GSH with N-ethylmaleimide prior to assay. The method was modified as follows: the DTNB concentration in the assay mixture was decreased from 0.6 to 0.15 mM to reduce the background color production, and the assay was carried out at 30°C instead of 20°C to increase sensitivity. Cell samples (2 x 10^6 cells/ml) were centrifuged at 0–5°C, resuspended in an equal volume of 0.9% NaCl solution, and protein was precipitated with 10% trichloroacetic acid (final concentration). The supernatants from the trichloroacetic acid precipitation were extracted with 5 equal volumes of (freshly opened) ether and stored frozen until assayed. The accuracy of the enzymatic assay was confirmed by chromatographic separation and quantification of low-molecular-weight thiols from DEM-treated cells as monobromobimane derivatives (30).

Protein thiols were assayed colorimetrically with DTNB (18) after extraction of cells with 2% SDS in 0.1 M Tris, pH 8.0, and filtration through Sephadex G-25 (equilibrated and eluted with 0.1% SDS in 10 mM phosphate buffer, pH 7.5), to remove low-molecular-weight thiols. This separation was found to be necessary as complete homogenates continued to reduce DTNB beyond the point where the reaction with isolated protein was complete (30 min), suggesting that an enzymatic reaction was contributing to color formation.

Binding of MIS Metabolites. Binding of [2-'4C]MIS to cellular macromolecules was determined by scintillation counting after extraction of cells with 2% SDS in 0.1 M Tris, pH 8.0, and overnight dialysis against 0.1% SDS in 10 mM phosphate buffer, pH 7.5. The possibility that counts might be lost during dialysis was excluded by comparison of results obtained by dialysis with results obtained by rapid gel (Sephadex G-25) filtration.

Cell Culture. CHO-To cells were grown in suspension culture in a modified minimum essential medium containing 10% fetal bovine serum, and were harvested by centrifugation. Cells were resuspended in water-jacketed Wheaton flasks with constant stirring at 37°C. Cell suspensions (2 x 10^6 cells/ml) were gassed for 1 hr with 5% CO_2 in either air or N_2 prior to addition of drugs, and were continually gassed throughout the experiment. Samples were taken with gas-equilibrated pipets and diluted into medium containing serum, for viability estimation by colony-forming ability, as described previously (38).

RESULTS

Kinetics of Glutathione Depletion by MIS and DEM. Hypoxic incubation of CHO cells with MIS at 37°C resulted in depletion of intracellular GS (Chart 1). The rate of glutathione depletion was dependent on MIS concentration. It should be noted that the results reflect the difference between GS depletion by MIS and GS resynthesis. The rate of resynthesis of GS in washed cells following depletion with either MIS or DEM resulted in restitutions of GS to control values within 3 to 6 hr of drug removal (data not shown). No effect of MIS on GS levels was seen under aerobic conditions at concentrations of MIS up to 5 mM, although concentrations of MIS (75 mM) which were toxic under aerobic conditions, depleted GS (Chart 1). Aerobic exposure to 75 mM MIS additionally resulted in oxidation of the remaining GS (GSSG was 60% of the total GS after 2 hr, compared to <1% GSSG in the controls). Exposure of hypoxic cells to 5 mM MIS for 2 hr did not affect the GSSG/GS ratio, even though it resulted in as much cytotoxicity as aerobic exposure to 75 mM MIS for 2 hr. This suggests the mechanisms of aerobic and hypoxic cytotoxicity may differ mechanistically.

DEM (0.2 mM for 1 hr) decreased GS concentrations in hypoxic CHO cells to 1 to 2% of control values, and did not result in loss of viability over a period of 8 hr at 37°C. The kinetics and concentration dependence of GS depletion by DEM are shown in Chart 2. A steady-state GS concentration was generally achieved after 1 hr of incubation with DEM with no alteration of the GSSG/GS ratio. Protein thiol levels were unaffected by 0.2 mM DEM (up to 4 hr of incubation at 37°C), although 1 mM DEM, which was cytotoxic after 4 hr of hypoxic treatment, did decrease the concentration of protein thiols (data not shown). Addition of GSH (3 mM) to the culture medium resulted in restoration of intracellular glutathione to control values within 15 min in cells which had been depleted of GS by either MIS or DEM. The intracellular location of GSH in GSH-restored cells was confirmed by retention of GSH following

![Chart 1. Depletion of intracellular GS in CHO cells in vitro by MIS. CHO-To cells (2 x 10^6 cells/ml) were incubated at 37°C with MIS under hypoxic (1 mM MIS (D); 2 mM MIS (I); 5 mM MIS (O)), and aerobic (5 mM MIS (C)); 75 mM MIS (B)) conditions, as described in "Materials and Methods." Samples were taken at the indicated times for GS assay. Data shown are individual measurements from several experiments. Values are corrected for increases (up to 20%) in control GS content during the course of the incubation. Control GS concentration was 2.58 ± 0.26 mM.](chart1.png)
Role of Glutathione in MIS Cytotoxicity

Cytotoxic action of MIS, as indicated by the end of the shoulder of the cytotoxicity curve (Chart 3), with little or no variability in these parameters between cell lines and even within one cell line on different occasions.

Depletion of GS with 0.2 mM DEM prior to treatment with MIS (5 mM) under hypoxic conditions resulted in a decrease in the shoulder of the cytotoxicity curve (Chart 3), with little or no effect on the exponential slope (the differences in slope of the 3 curves shown in Chart 3 are only significant at the 80% confidence level). Restoration of GS to control levels prior to addition of MIS resulted in a restoration of the shoulder, and the prolongation of the shoulder beyond that of the controls. Similar results were seen at 1 and 2 mM MIS (Table 2). Addition of GSH to the medium also prolongs the shoulder of the MIS cytotoxicity curve in cells which were not pretreated with DEM (data not shown), in agreement with previous results of Taylor and Rauth (39). All experiments were carried out in the presence of serum. Control experiments in the absence of serum indicated that serum had no effect on the sensitivity of CHO-To cells to MIS under the conditions used (data not shown).

Covalent Binding of MIS Metabolites. Binding of [2-14C]MIS to cellular macromolecules was not appreciably increased by centrifugation through silicone oil under conditions which completely excluded extracellular fluid from the cell pellet.

Hypoxic Cytotoxicity of MIS. The kinetics of GS depletion by MIS was similar to the kinetics of loss of resistance to the cytotoxic action of MIS, as indicated by the end of the shoulder of the MIS cytotoxicity curve. This is shown in Table 1. The extrapolation of the exponential portion of the semilogarithmic plot of survival versus incubation time to the 100% survival level ($D_0$), which is a measure of the shoulder of the curve, was consistently greater than the time required to deplete GS to 20% of control values over the range of 1 to 5 mM MIS, despite variations in sensitivity.

Table 1

<table>
<thead>
<tr>
<th>MIS concentration (mM)</th>
<th>GS depleted</th>
<th>GS restored</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.0 ± 0.2(2)</td>
<td>2.8 ± 0.4 (2)</td>
</tr>
<tr>
<td>2</td>
<td>2.3 ± 0.1(5)</td>
<td>1.2 ± 0.1 (4)</td>
</tr>
<tr>
<td>5</td>
<td>1.6 ± 0.1 (6)</td>
<td>0.6 ± 0.07 (7)</td>
</tr>
<tr>
<td>2.3 ± 0.1(3)</td>
<td>2.4 ± 0.3 (3)</td>
<td></td>
</tr>
</tbody>
</table>

* DEM (2 × 10^-4 mM) was added 1 hr before MIS; this resulted in depletion of glutathione to 1 to 3% of control values at the time of MIS addition. Control GS concentration was 2.58 ± 0.26 mM.

** $D_0$, time (hr) required for MIS to deplete intracellular glutathione to 20% of control values; $D_0$, time (hr) of MIS treatment corresponding to 100% cell survival on the extrapolation of the exponential portion of the survival curve.

*** CHO-To cells were grown and treated in suspension (2 × 10^6 cells/ml) in a-modified minimum essential medium containing 10% fetal bovine serum.

Table 2

<table>
<thead>
<tr>
<th>MIS concentration (mM)</th>
<th>Not pretreated</th>
<th>GS depleted</th>
<th>GS restored</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.0 ± 0.2(2)</td>
<td>2.8 ± 0.4 (2)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2.3 ± 0.1 (5)</td>
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</tr>
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* DEM (2 × 10^-4 mM) was added 1 hr before MIS; this resulted in depletion of glutathione to 1 to 3% of control values at the time of MIS addition. Control GS concentration was 2.58 ± 0.26 mM.

** $D_0$, time (hr) on MIS treatment corresponding to 100% cell survival on the extrapolation of the exponential portion of the survival curve (Chart 3 shows survival curves for 5 mM MIS).

*** GS (3 mM) was added after DEM pretreatment, as described above. This resulted in restoration of intracellular GS levels to control values at the time of MIS addition. Restored GS was not depleted during MIS treatment.

**** Average ± S.E. of separate experiments.

***** Numbers in parentheses, number of experiments.
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(29) nitroaromatic derivatives have been shown to react with hypoxic conditions, as described in “Materials and Methods,” after a 1-hr pretreatment with 0.2 mM DEM (○). •, cells treated with MIS only. Samples were taken at indicated times for dialysis and scintillation counting. Data shown are from a representative experiment. A preliminary report of these data was published (Brown, J. M. The mechanism of cytotoxicity and chemosensitization by mitomycin and other nitroimidazoles. Int. J. Radiat. Oncol. Biol. Phys.; 8: 675-682, 1982).

 prior depletion of GS with DEM (Chart 4). In fact, after a 2-hr incubation with MIS, the extent of binding of 14C-labeled MIS metabolites was less in DEM-pretreated cells than in cells treated with MIS alone, despite a difference in survival of 3 orders of magnitude (Charts 3 and 4). The rate of MIS binding to macromolecules decreased in both cases at exposure times corresponding to cell survivals of less than 1%, suggesting that extensive cell killing by MIS is accompanied by inhibition of further drug reduction.

**DISCUSSION**

The loss of GS observed in hypoxic CHO cells exposed to MIS (Chart 1) appears to be a result of the reaction of GSH with reduced metabolites of MIS, since no loss of GS is observed with 5 mM MIS in aerated cells, and GSH does not react at an appreciable rate with MIS in a pure chemical system, in the presence or absence of glutathione S-transferase (data not shown). Various nitroso (17, 19, 20, 29) and hydroxylamine (29) nitroaromatic derivatives have been shown to react with GSH to produce stable adducts, among other products. Such adduct formation would remove reactive intermediates which might otherwise bind covalently to cellular macromolecules (25, 42). The data shown in Chart 4 for the binding of MIS metabolites to macromolecules in CHO cells which were not pretreated prior to exposure to MIS are consistent with such a protective mechanism. The initial rate of MIS binding to macromolecules is lower than the maximal rate observed when GS is depleted (after 2 hr of MIS treatment). This difference is statistically significant at the 99% confidence level. The difference between the amount of MIS observed to bind to cellular macromolecules and the amount that would be calculated to bind if binding occurred at the maximal rate all along (1.8 nmol/10^6 cells) may be an indication of the extent to which GSH was able to protect against binding, by intercepting reactive intermediates. Since the amount of GS depleted was 3.6 nmol/10^6 cells, it would appear that approximately 50% of the metabolites intercepted by GSH would have bound to macromolecules, had they not been intercepted. Depletion of GS with DEM prior to exposure to MIS, however, did not significantly accelerate the binding of MIS metabolites to macromolecules, possibly because the rate of generation of reduced metabolites was less under these conditions.

The important finding with regard to binding of MIS metabolites to cellular macromolecules is that depletion of GS by pretreatment with DEM greatly enhances the cytotoxicity of MIS, without affecting the initial rate of binding. Comparison of binding of MIS to macromolecules (Chart 4) with the extent of cell killing (Chart 3) 2 hr after MIS addition, when the level of MIS binding is approximately the same in DEM-pretreated cells as in cells which had not been pretreated, leads to the conclusion that the amount of binding of MIS metabolites to macromolecules does not correlate with the extent of cytotoxicity, since there is a 1000-fold difference in cell survival, with no difference in binding. Although DEM might have enhanced the cytotoxicity of MIS by a mechanism other than depletion of GS, the reversal of the effect of DEM by GS restoration (Chart 3) indicates it is indeed GS depletion that accounts for this effect. It would therefore appear that GSH plays another role in protecting against MIS cytotoxicity, which is more important than removing those metabolites of MIS which bind to cellular macromolecules.

The precise pathway for the reductive metabolism of MIS in hypoxic cells is not completely understood. Several free radical intermediates have been suggested to occur during reduction of the nitro group, on the basis of the metabolism of 4-nitroquinoline N-oxide (2, 43), although only the nitro anion radical has been directly identified as a metabolite of MIS. There are also indications that ring scission occurs (21), and this could provide an additional source of free radicals. The protection against the toxicity of MIS to hypoxic cells observed with the stable free radical triacetoneamine-N-oxyl (34) might also be an indication that free radical intermediates of MIS metabolism are toxic, since radical-radical interactions could result in their detoxification. Since thiols are highly reactive toward free radicals, donating hydrogen to most carbon-, oxygen-, and nitrogen-centered radicals (28), this could explain the protection by GSH against MIS cytotoxicity seen in this study. Thus we would like to propose that the cytotoxicity of MIS to hypoxic cells is mainly a result of the action of free radicals generated during its metabolism, and that GSH protects against MIS cytotoxicity by reducing these radicals by hydrogen donation.

The reactions proposed are summarized in Chart 5. Reduced intermediates of MIS metabolism are indicated as TH (non-radical intermediates) or X- (radical intermediates) and are shown to react by different mechanisms with cellular targets (TH) or with GSH. Electrophilic attack by XH results in the formation of covalent adducts (TX or GSX), while hydrogen abstraction results in free radical formation (T- and GS-). GSH competes with cellular targets for MIS metabolites. Inefficient competition for electrophilic attack allows TX formation but does not result in cell death. Efficient competition for free radical intermediates results in protection of TH from free radical attack. Removal of GSH allows free radical attack on cellular targets, resulting in cell death. GSH can also have a protective effect by restoring target radicals (T-) to their original state (TH).

It should be noted that a similar mechanism, not involving free radicals, is also possible. The interaction between GSH and nitroso aromatic compounds can result in the formation of
REFERENCES

ACKNOWLEDGMENTS

Role of Glutathione in MIS Cytotoxicity


* E. A. Bump and J. M. Brown, unpublished observations.
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